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IS 8268 (2001): Rhizobium Inoculants [FAD 7: Soil Quality and Gertilizers]

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भारतीय मानक
राइजोबियम जीवाणु कल्पर — विशिष्टि
(दूसरा पुनरीक्षण)

Indian Standard

RHIZOBIUM INOCULANTS — SPECIFICATION
(Second Revision)

ICS 65.080

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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR ST.
NEW DELHI 110002

AMENDMENT NO. 1 JUNE 2008
TO
IS 8268 : 2001 RHIZOBIUM INOCULANTS —
SPECIFICATION

(Second Revision)

(Page 1, clause 4.2.1, line 9) — Substitute 'D-4.1.6' for 'D-4.1.5'.

(Page 1, clause 4.9, line 2) — Substitute '30-35 percent (m/m)' for '30-40 percent (m/m)'.

(Page 5, Annex B) — Substitute the following for the existing text:

ANNEX B

(Clause 4.5)

DETERMINATION OF pH AND MOISTURE PERCENTAGE

B-1 pH

B-1.1 Make 20 g of the RI into a suspension in 50 ml of distilled water and shake on a rotary shaker for 2 h, filter on Whatman No. 1 filter paper or equivalent under vacuum using a Buchner funnel. Determine pH of the filtrate in a pH meter.

B-2 DETERMINATION OF MOISTURE OF BIOFERTILIZER PACKETS (METHOD)

B-2.1 Heat 10 g of sample for 12 - 16 hours in an air oven at 100 - 105°C. Cool in a desiccator and weigh. The loss in weight represents the moisture. Calculate the moisture percentage on air dry weight basis, by multiplying the loss in weight by ten.

(FAD 7)

FOREWORD

This Indian Standard was adopted by the Bureau of Indian Standards, after the draft finalized by the Soil Quality and Improvement Sectional Committee had been approved by the Food and Agriculture Division Council.

Bacteria of the genus *Rhizobium* (plural *Rhizobia*) form nodules on roots of certain leguminous plants and fix atmospheric nitrogen. These bacteria, although present in most soil types, vary in number and effectiveness in nodulation and nitrogen fixation, hence wherever necessary seed inoculation is practised to ensure adequate population of effective strains of the required rhizobia in the root zone so as to improve nodulation, nitrogen fixation, crop growth and yield of leguminous crops. Leguminous crops not only depend on nitrogen fixed biologically in root nodules for their growth but also may add considerable amount of nitrogen into soil for the benefit of a subsequent crop, which is one of the scientific basis in the agricultural practice of crop rotation. With suitable *Rhizobium* inoculation, leguminous crops can be raised successfully without resorting to large application of inorganic nitrogenous fertilizers, except for starter doses up to 20 kgN/ha. Many brands of *Rhizobium* inoculants are marketed in the country and they have been found to vary in quality. Hence, need was felt or an Indian Standard not only to test the quality of inoculants in order to provide the farmers with certified inoculants but also to help the producers improve the quality of their products.

This standard was published in 1976 and first revised in 1986 where number of changes were made. The second revision incorporates latest technological developments in this field and update methods of tests. In this revision a number of changes were made and prominent amongst them are as follows:

- a) Serological test for checking the presence of required strains in the *Rhizobium* inoculants (RI) has been retained.
- b) Dilution at which RI shall have no contamination, has been changed.
- c) Viability strains of cells be counted by MPN method.
- d) Moisture percent test has been added in the form of fribal (moist).
- e) Packing material requirements has been changed.
- f) Self life of Azotobacter chroococcum inoculant has been included.
- g) pH value of RI has been changed.

It is obligatory on the part of the manufacturers to employ qualified soil microbiologists or general microbiologists or agricultural graduates or graduates in biology trained in soil microbiology on their staff. Manufacturers shall also maintain a quality control laboratory capable of carrying out the tests according to this specification.

The composition of the Committee responsible for the formulation of this standard is given in Annex G.

For the purpose of deciding whether a particular requirement of this standard is complied with, the final value, observed or calculated, expressing the result of a test or analysis, shall be rounded off in accordance with IS 2 : 1960 'Rules for rounding off numerical values (revised)'. The number of significant places retained in the rounded off value should be the same as that of the specified value in this standard.

Indian Standard

RHIZOBIUM INOCULANTS — SPECIFICATION

(Second Revision)

1 SCOPE

This standard prescribes the requirements and methods of sampling and test for *Rhizobium* inoculants.

2 REFERENCES

The Indian Standards listed below contain provisions which through reference in this text, constitutes provision of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated.

<i>IS No.</i>	<i>Title</i>
1070 : 1992	Reagent grade water (<i>third revision</i>)
6850 : 1973	Agar, microbiological grade
6853 : 1973	Peptone, microbiological grade
7004 : 1973	Yeast extract, microbiological grade

3 TERMINOLOGY

3.1 For the purpose of this standard, the following definitions shall apply.

3.2 *Rhizobium* Inoculants (RI)

RI is a product having a large population of a strain or a combination of several strains of *Rhizobium* for a particular leguminous plant or a group of leguminous plants which shall produce efficient root nodules on the relevant plants and thereby enhance nitrogen fixation, crop growth and yield.

3.3 Group of Leguminous Plants

Plants which are nodulated effectively by a given strain of *Rhizobium* inoculants (RI).

4 REQUIREMENTS

4.1 RI shall be carrier-based, the colour depending on the colour of the carrier.

4.2 RI shall contain a minimum of 10^7 viable *Rhizobium* cells/g of the carrier on dry-mass basis till 6 months expiry period from the date of manufacture. The number shall be counted by the plate count method as given in Annex A.

4.2.1 The presence of the required strain shall be checked serologically as outlined in Annex D. If a single strain is used and no contaminants are present then the whole contents of the plate can be suspended in saline and tested. With more than one strain, or contaminants present, 5 single colonies must be picked, grown on yeast Extract Mannitol Agar (A-2.2) and checked individually as outlined in D-4.1.5. At least one of the five isolates must agglutinate with one of the antisera. Failure of all colonies to agglutinate, when tested against all the antisera of all the strains supposed to be present, results in rejection of the batch. This test to be retained for confirmation of the strains.

4.3 RI shall have a maximum of six months' expiry period from the date of manufacture.

4.4 RI shall have no contamination with other micro-organisms at 10^5 dilution when counted as given in Annex A.

4.5 The pH of RI shall be between 6.5 and 7.5 when tested as given in Annex B.

4.6 RI shall show effective nodulation on all those species and/or cultivars listed on the packet before the expiry date when tested by the method given in Annex C. If good effective pink nodulation is obtainable in the inoculated species together with total absence or sometimes presence of stray nodules in the controls, it should be concluded that RI contains effective *Rhizobium*. The total dry mass of inoculated plants shall be significantly higher than that of the uninoculated controls and at least 50 percent more than the controls.

4.7 The carrier material such as peat, lignite, peat-soil, humus or similar material favouring growth to be neutralized with calcium carbonate and sterilized shall be in the form of a powder capable of passing through 150 to 212 micron (72 to 100 mesh) IS sieve.

4.8 Specified mother culture be obtained from any recognized institution maintaining the mother cultures. The manufacturer may control the quality of the broth as given in Annex D.

4.9 Moisture

The RI carrier with 30-40 percent moisture content

5 PACKING, MARKING AND STORAGE

5.1 Packing

RI shall be packed in packaging material of low density polyethylene/polypropylene bags thickness of which shall be 75-100 micron minimum.

5.2 Marking

Each packet shall be marked legibly to give the following information:

- a) Name of the product, specifically as *Rhizobium* inoculant;
- b) Leguminous crop for which intended;
- c) Name and address of the manufacturer;
- d) Type of the carrier;
- e) Batch or Code number;
- f) Date of manufacture;
- g) Date of expiry (agreed between the manufacturer and the purchaser subject to minimum 6 months from the date of manufacture);
- h) Net quantity and the area meant for;
- i) Storage instructions worded as under: 'STORE IN COOL PLACE AWAY FROM DIRECT SUN AND HEAT'.
- k) Any other information.

5.2.1 Item (b), (f) and (g) shall be printed on a coloured ink background.

5.2.2 Directions for the use of RI shall be printed briefly on the packet as given in Annex E. A separate pamphlet may preferably be given with it.

5.2.3 BIS Certification Marking

The product may also be marked with the Standard Mark.

5.2.3.1 The use of the Standard Mark is governed by the provisions of the *Bureau of Indian Standards Act, 1986* and the Rules and Regulations made thereunder. The details of conditions under which the licence for the use of the Standard Mark may be granted to manufacturers or producers may be obtained from the Bureau of Indian Standards.

5.3 Storage

RI shall be stored by the manufacturer in a cool and dry place away from direct heat preferably at a temperature of 15°C to 30°C. It shall also be the duty of the manufacturer to instruct the retailers and, in turn, the users about the precautions to be taken during storage.

6 SAMPLING

6.1 The method of drawing representative samples of RI from different batches and the criteria for conformity shall be as given in Annex F.

7 TESTS

7.1 Tests shall be carried out by the methods prescribed in 4.2 to 4.6.

7.2 Unless otherwise specified, quality reagents, chemicals and distilled water (*see IS 1070*) shall be employed in tests.

NOTE — 'Pure chemicals' shall mean chemicals that do not contain impurities which affect experimental results.

ANNEX A

(Clauses 4.2 and 4.4)

DETERMINATION OF NUMBER OF *RHIZOBIUM* CELLS

A-1 APPARATUS

A-1.1 Pipettes — Graduated, 1-ml, 10-ml.

A-1.2 Dilution Bottles or Conical Flasks

A-1.3 Petri Dishes — Clear uniform flat-bottomed.

A-1.4 Hot-Air Oven — Capable of giving uniform and adequate temperatures equipped with a thermometer, calibrated to read up to 250°C, and with vents suitably located to assure prompt and uniform heating.

A-1.5 Autoclave

A-1.6 Incubator

A-1.7 Hand Tally or Mechanical Counting Device

A-1.8 pH Meter

A-2 REAGENTS

A-2.1 Congo Red — One percent aqueous solution.

A-2.2 Medium — Use a plating medium of the following composition:

Agar (<i>see IS 6850</i>)	20 g
Yeast extract (<i>see IS 7004</i>)	1 g
Mannitol	10 g
Potassium hydrogen phosphate (K ₂ HPO ₄)	0.5 g
Magnesium sulphate (MgSO ₄ .7H ₂ O)	0.2 g
Sodium chloride (NaCl)	0.1 g
Congo red	2.5 ml
Distilled water	10.00 ml
pH	7.0

A-2.3 Sterilizing and Preparation Procedure for Plates

A-2.3.1 Sterilize sampling and plating equipment with dry heat in a hot-air oven, at not less than 160°C for not less than 2 h.

A-2.3.2 Sterilize the medium by autoclaving at 120°C for 20 min. To permit passage of steam into and from

closed containers when autoclaved, keep stoppers slightly loosened, air from within the chamber of the sterilizer should be ejected allowing steam pressure to rise.

A-2.3.3 Prepare growth medium in accordance with the composition indicated in A-2-2.

A-2.3.4 Melt required amount of medium in boiling water or by exposure to flowing steam in a partially closed container, but avoid prolonged exposure to unnecessarily high temperature during and after melting. Melt enough medium which will be used within 3 h. Resterilization of medium may cause partial precipitation of ingredients.

A-2.3.5 When holding time is less than 30 min, promptly cool the melted medium to about 45°C and store, until used, in a water-bath or incubator at 43 to 45°C and introduce 12 to 15 ml of liquefied medium or appropriate quantity depending on size of petri-dish at 42 to 44°C into each plate. Gently lift the cover of the dish just enough to pour in the medium. Sterilize the lips of media containers by exposure to flame (a) immediately before pouring; (b) periodically during pouring; and (c) when pouring is complete for each batch of plates, if portions of melted medium remain in containers and are to be used without subsequent sterilization for pouring additional plates.

A-2.3.6 By rotating and tilting the dish and without splashing the medium over edge, spread the medium evenly over the bottom plate. Provide conditions so that the medium solidifies with reasonable promptness (5 to 10 min) before removing the plates from level surface.

A-3 PREPARATION OF SERIAL DILUTIONS FOR PLATE COUNTS

A-3.1 Dispense 30 g of RI in 300 ml of sterile distilled or demineralized water and shake for 10 min on a reciprocal shaker or homogenizer making serial dilutions up to 10¹⁰. Take 0.2 ml aliquots of 10⁶ to 10⁸ dilution using sterile pipettes and deliver to petri-dishes containing set medium as even in A-2.2 and spread it uniformly. Invert the plates and promptly place them in the incubator.

A-4 INCUBATION OF PLATES**A-4.1 Label the plates and incubate at $28 \pm 2^\circ\text{C}$ for**

3 to 5 days for fast growing rhizobia and 5 to 10 days for slow-growing ones. The common species of fast and slow-growing rhizobia are given below:

a) Fast-Growing Rhizobia

<i>Rhizobium sp.</i>	<i>Hosts</i>	
	Botanical Name	Common Name
<i>Rhizobium metiloti</i> (Medic-rhizobia)	<i>Medicago sativa</i> <i>Trigonella foenum-graecum</i>	Lucerne Fenugreek
<i>Rhizobium trifolii</i> (Clover-rhizobia)	<i>Trifolium alexandrinum</i> <i>Trifolium sp.</i>	Egyptian clover Clovers
<i>Rhizobium leguminosarum</i> (Pea-rhizobia)	<i>Lathyrus sativus</i> <i>Lens culinaris</i> <i>Pisum sativum</i> <i>Vicia sativa</i>	Grass pea, Khesari Lentil Pea Common vetch
<i>Rhizobium phaseoli</i> (Bean-rhizobia)	<i>Phaseolus multiflorus</i> <i>P.vulgaris</i>	Bean Kindney bean, French bean
<i>Rhizobium sp.</i>	<i>Cicer arietinum</i>	Chickpea, Bengal Gram

b) Slow-Growing Rhizobia

<i>Rhizobium lupini</i> (Lupin-rhizobia)	<i>Lupinus alba</i>	<i>White lupines</i>
<i>Rhizobium japonicum</i> (Soybean-group)	(<i>Lupinus sp.</i>) <i>Glycine max</i>	Lupines Soybean
<i>Rhizobium sp.</i> (Cowpea Miscellany) (see Note)	<i>Crotalaria juncea</i> <i>Cyemopsis tetragonoloba</i> <i>Arachis hypogaea</i> <i>Lespedeza sericea</i> <i>Canavalia gladiata</i> <i>Lablab purpureus</i> <i>Macrotyloma uniflorum</i> <i>Phaseolus acutifolius</i> <i>Vigna mungo</i> <i>V. radiata</i> <i>V. sinensis</i> <i>Cajanus cajan</i> <i>Calopogonium mucunoides</i>	Sunn hemp Cluster bean (guar) Peanut Lespedeza (Kudzu) Jack bean or sword bean Lablab Horse gram (Kulthi) Moth bean Black gram Green gram Cowpea Red gram Calopogonium, Calopo

NOTE — Several *Rhizobium* strains of cowpea miscellany (*Rhizobium sp.*) may be fast growing.

A-4.2 Colony Counting Aids

Count the colonies with the aid of magnifying lens under uniform and properly controlled, artificial illumination. Use a colony counter, equipped with a

guide plate and rules in centimetre square. Record the total number of colonies with the hand tally. Avoid mistaking particles of undissolved medium or precipitated matter in plates for pin-point colonies. To distinguish colonies from dirt, specks and other

foreign matter, examine doubtful objects carefully.

A-4.3 Count all plates but consider for the purpose of calculation plates showing more than 30 and less than 300 colonies per plate. Disregard colonies which absorb congo red and stand out as reddish colonies.

Rhizobium stands out as white, translucent, glistening and elevated colonies. Count such colony numbers and calculate figures in terms of per litre, of carrier. Also check for freedom from contamination at 10⁸ dilution (see 4.2).

ANNEX B

(Clause 4.5)

DETERMINATION OF pH

B-1 Make 20 g of the RI into a suspension in 50 ml of distilled water and shake on a rotary shaker for 2 h, filter on Whatman No. 1 filter paper or equivalent under

vacuum using a Buncher funnel. Determine pH of the filtrate in a pH meter.

ANNEX C

(Clause 4.6)

TEST FOR NODULATION

C-1 POT CULTURE TEST

C-1.1 Plant Nutrient Solution

Composition	Concentration	g/l
a) Potassium chloride	0.001 M	0.0745
b) Potassium hydrogen phosphate (K_2HPO_4)	0.001 M	0.175
c) Calcium sulphate ($CaSO_4 \cdot 2H_2O$)	0.002 M	0.344
d) Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.001 M	0.246
e) Trace elements solution:		
1) Copper sulphate ($CuSO_4 \cdot 5H_2O$)	0.01 mg/kg	0.78
2) Zinc sulphate ($ZnSO_4 \cdot 7H_2O$)	0.025 mg/kg	2.22
3) Manganese sulphate ($MnSO_4 \cdot 4H_2O$)	0.25 mg/kg	2.03
4) Ammonium molybdate [$(NH_4)_6MO_3O_{12} \cdot 4H_2O$]	0.0025 mg/kg	0.01
5) Boric acid (H_3BO_4)	0.125 mg/kg	1.43

Prepare the solution No. (e) consisting of trace elements in one litre of stock solution and add final nutrient solution at the rate of 0.5 ml per litre.

Composition

Concentration g/l

f) Iron solution: g/100 ml

1) Ferrous sulphate	5
2) Citric acid	5

Prepare the solution No. (f) as 100 ml of stock solution and add final nutrient solution at the rate of 0.5 ml per litre.

C-1.1.1 Preparation

Prepare the nutrient solution by weighing out substances (a), (b) and (d) and dissolving them in a litre of water. To this solution add 0.5 ml of trace elements solution and 0.5 ml of iron solution. Grind in a mortar 0.344 g of calcium sulphate (c) to a fine consistency and add to the final nutrient solution. Autoclave the nutrient solution thus prepared at 120°C for 20 min.

NOTES

1 The nutrient solution may be prepared in the tap water provided the water is soft.

2 The nutrient solution should be shaken well to disperse calcium sulphate before dispensing.

3 If the solution is made ...
is about ...

autoclaving ... standing to about 58
However, there is no need to adjust pH. For most tropical legumes, pH of about 6.0 is adequate.

C-1.2 Procedure

C-1.2.1 Immerse the seeds in 95 percent alcohol and follow by surface-sterilization in freshly prepared chlorine water (for 15 to 20 min) or 0.1 percent mercuric chloride solution for 3 min in a suitable container such as a screw-capped bottle or a test-tube with a rubber hung. In case of seeds with tough seed coat, concentrated sulphuric acid may be used as a surface sterilant for 20 to 30 min. It is recommended that the seeds should be placed overnight in a desiccator containing calcium chloride before surface sterilization with sulphuric acid. Pour out the sterilant and wash the seeds in several changes of sterile water (at least ten times) to get rid of the sterilant. Fill earthenware or glazed pots with soil (2 parts soil and 1 part washed coarse sand) (pH 6 to 7) and autoclave for 2 h at 120°C. After two days incubation at room temperature, repeat autoclaving to ensure complete sterility of soil. Inoculate surface-sterilized seeds with a water slurry of the inoculant taken from a culture packet (15 to 100 g seeds per gram of inoculant depending on the size of the seed) and sow the seeds. Keep a set of pots with uninoculated seeds as control and also a set of pots with ammonium nitrate at the rate of 100 kgN/ha as control aid incubate them in a pot-culture house during appropriate seasons for appropriate plants, taking care to separate the inoculated pots from the control pots. If growth rooms or cabinets having facilities to adjust temperature and light are available, the pots may be incubated in such controlled environmental conditions. Sterilize the nutrient

solution at 120°C for 20 min and irrigate each pot once to the moisture holding capacity of soil. Subsequently, water the seedling periodically with sterilized water preferably through a plastic tube, taking care to prevent splashing of water from inoculated pots to uninoculated ones. Maintain required number of replicated pots (4 to 6) for each botanical species for statistical analysis.

C-1.2.2 After two to three weeks of growth, thin down the number of plants in each pot to four uniform plants. At the end of 6 to 8 weeks, take one set of pots from both the control and inoculated series and, separate the plants carefully from the soil under slow-running water. Obtain data on the number, colour (effective nodules are pink or red) and mass of nodules. At the end of 6 to 8 weeks, harvest the shoot system, dry at 60°C for 48 h and determine dry mass. For the above purpose, maintain adequate replications of pots (4 to 16).

C-1.2.3 Record the nodulation data regarding formation of pink colour of nodules as revealed visually when nodules are cut open by a razor blade. After computing the data, based on the dry mass of plants and nodulation data decide the effectiveness of cultures. If good effective pink modulation is obtainable in inoculated plants together with local absence or sometimes presence of stray nodules in controls and if there is a 50 percent increase in the dry mass of plants over the uninoculated control without nitrate, it may be concluded that the culture is of the required quality.

ANNEX D

(*Clauses 4.2.1 and 4.8*)

**GUIDELINES ON MAINTENANCE AND PREPARATION OF CULTURE AND
QUALITY CONTROL AT BROTH STAGE**

D-1 MAINTENANCE OF PURE CULTURES

D-1.1 Maintain pure cultures of rhizobia on yeast extract mannitol agar (YEMA) slants of the following composition:

Mannitol	10.0 g
Potassium hydrogen phosphate (K ₂ HPO ₄)	0.5 g
Magnesium sulphate (MgSO ₄ .7H ₂ O)	0.2 g
Sodium chloride (NaCl)	0.1 g
Calcium carbonate (CaCO ₃)	1.0 g
Yeast extract (see IS 7004)	1.0 g

Agar (see IS 6850) 18.0 g

Distilled water 1 litre

pH 6.8-7.0

D-1.2 Transfer a loopful of the pure culture to each of the agar slants aseptically in an inoculation room and incubate at 28 ± 2°C for 3 to 10 days depending upon the species of *Rhizobium*. Always keep pure cultures at 4°C.

D-2 PREPARATION OF INOCULUM CULTURES

D-2.1 Prepare yeast extract mannitol broth of the composition as given in D-1.1 minus the agar.

D-2.2 Weigh the ingredients, pool, dissolve in required amount of water and make upto known volume. Dispense 100 ml aliquots of the broth into 250 ml conical flasks and plug with non-absorbent cotton. Autoclave the flasks at 120°C for 20 min, cool and inoculate with the required *Rhizobium* as given in D-2.2.1.

D-2.2.1 Examine a 4 to 6 days old pure culture visually and by Gram staining test for freedom from contamination and transfer approximately 2 to 3 ml of sterile broth aseptically into it. Scrape the bacterial growth with the help of an inoculation needle and transfer the resulting bacterial suspension into a 250 ml conical flask containing the broth. Incubate the flasks at 28 ± 2°C on a rotary shaker for 2 to 6 days depending upon the species of the *Rhizobium*.

D-3 PREPARATION OF MASS CULTURE

D-3.1 Form a battery of conical flasks or suitable fermentors each containing required amounts of broth (see D-2.1) and proceed for sterilization (see D-2.2). Into each one of these flasks, transfer requisite quantity of inoculum culture aseptically and incubate the flasks preferably on a rotary shaker at 28 ± 2°C for 2 to 6 days depending on the species of the *Rhizobium*.

NOTE — If the medium (broth) is formulated in a fermentor the amount of inoculum culture to be transferred into the fermentor is variable depending on the size of the fermentor but the ratio between the inoculum culture and the medium formulated in the fermentor is recommended as 1 : 20 (inoculum : quality of broth) in the fermentor.

D-4 QUALITY CONTROL TESTS RECOMMENDED AT BROTH STAGE

D-4.1 Qualitative Tests

D-4.1.1 Check for freedom from visible contaminants.

D-4.1.2 The pH of the bacterial broth shall normally be between 6.5 and 7.5.

D-4.1.3 Smear and Gram Stain

D-4.1.3.1 Reagents

a) *Ammonium oxalate crystal violet stain* — Weigh 0.2 g of crystal violet and dissolve in 20 ml of 95-percent ethyl alcohol. Dissolve separately 0.8 g of ammonium oxalate in 80 ml of distilled water. Mix the two solutions and filter through a filter paper.

b) *Iodine solution*

Iodine	1.00 g
Potassium iodide	2.00 g
Distilled water	300 ml

Weigh the ingredients and dissolve in water. Filter through a filter paper.

c) *Erythrosine*

Erythrosine	1.00 g
Phenol	5.00 g
Distilled water	100 ml

Weigh the ingredients, dissolve in distilled water and filter through a filter paper.

D-4.1.3.2 Procedure

Prepare a smear on a clean microscope slide, fix over a flame by gentle and intermittent heating, air cool and flood with ammonium oxalate crystal violet stain for 1 min. After removing the excess of ammonium oxalate crystal violet, wash the slide under a gentle stream of running tap-water. Flood the slide with iodine solution for half-a-minute, remove excess stain, wash with 95-percent ethyl alcohol and finally wash under a gentle stream of running tap-water. Flood the slide with erythrosine stain for about 3 min, wash under a gentle stream of running tap-water and dry between the folds of a filter paper. Examine the slide under a compound microscope using an oil immersion objective.

NOTE — A smear prepared from undiluted broth should be free from Gram positive cells. The presence of a few Gram positive cells in occasional fields which may be due to dead cells in the medium may be disregarded.

D-4.1.4 Absence of Growth on Glucose-Peptone Agar

The composition of the glucose-peptone agar is as follows:

Glucose	10.0 g
Peptone (see IS 6853)	20.0 g
Sodium chloride (NaCl)	5.0 g
Agar (IS 6850)	15.0 g
Distilled water	1 000 ml
Bromocresol purple	10 ml of 1.6-percent ethyl alcohol solution
pH	7.2

NOTE — When a loopful of the broth is streaked into this medium and incubated at 28 ± 2°C for 24 h, the purple-violet colour of the medium (due to the indicator bromocresol purple) shall not change. If the colour changes to yellow (acidic reaction) or blue (alkaline reaction), the broth is grossly contaminated. Hence, the broth should be rejected.

D-4.1.5 Streak

Congo Red

When a loopful of broths containing the species

plate of this medium and incubated at $28 \pm 2^\circ\text{C}$ for 3 to 10 days, it shall show colonies of bacteria with growth characteristics same as that of the pure culture used in the preparation of the broth. Otherwise, the broth should be rejected.

D-4.1.6 Agglutination with Specific Antiserum

D-4.1.6.1 Preparation of specific antiserum

Grow the pure culture of *Rhizobium* from which the broth is prepared for making inoculants, on YEMA in a petri-dish or in cotton-wool protected bottle-flats at $28 \pm 2^\circ\text{C}$ for 2 to 6 days, depending on the species of *Rhizobium*. Harvest the cells in physiological saline (0.85-percent NaCl), centrifuge at 15 000 rev/min and obtain a pellet. Suspend the pellet in minimum amount of physiological saline. This suspension will serve as an 'antigen'. Mix 1 ml of the above antigen (containing approximately 10 to 15 $\mu\text{g}/\text{ml}$ of protein or 10^6 cells/ml) with 0.5 ml of an adjuvant of microbiological grade or, sterilized paraffin oil and inject intramuscularly into a rabbit, on the upper part of the hind legs. Repeat such injection three times at 7-days intervals. On the twentyeighth day after the first injection, inject 1 ml of the antigen without the adjuvant intravenously through the marginal vein of the ear. After 7 days of the last injection, bleed the rabbit through the marginal ear vein to collect blood in a beaker. Allow the blood to coagulate at 35°C for an hour and store in a refrigerator overnight to obtain a clear straw-coloured supernatant which should be centrifuged at 3 000 rev/min for 5 min to remove sediments, if any. This will be the specific antiserum which should give definite agglutination with the antigen injected and have minimum titre of 3 200. Store the antiserum in a deep freeze adding sodium azide (NaN_3) (0.025 percent) and dispensing in small quantities in several vials. Antisera may also be Lyophilized and stored at 4°C .

D-4.1.6.2 Estimation of titre value

From 0.1 ml of a specific antiserum prepare in test tubes (75 \times 15 mm dia) a range of double-fold serial dilutions from 1/100 to 1/6 400 as given below using physiological saline:

Tube	1	2	3	4	5	6	7
Volume of saline, ml	4.9	2.5	2.5	2.5	2.5	2.5	2.5
Volume of serum (or volume of serum in ml from previous dilution)	0.1	2.5	2.5	2.5	2.5	2.5	2.5

(1) (2) (3) (4) (5)-(6) (7)
discard

Final dilution 100 200 400 800 1 600 3 200 6 400
after adding
2.5 ml of
antigen
suspension

NOTE — Figures in parentheses show the tube number from which diluted serum suspension should be taken.

D-4.1.6.3 Also keep a saline control. Add in each tube 2.5 ml aliquots (that is, the amount equivalent to the antiserum suspension as in each tube) of antigen suspension (10^5 to 10^7 cells/ml) or broth (in case of broth test). Mix the mixture in each tube (by Pasteur pipette) and allow to settle for 4 h at 37 or 55°C in a water-bath and afterwards overnight in a refrigerator. Observe the agglutination. Take reciprocal of the highest dilution in which agglutination occurs, as the titre value of the antiserum tested. In the case of broth, agglutination should occur at 100 or 200 litre. On the other hand, saline control should always show turbidity. This sample test will permit detection of gross contamination or substitution of a different *Rhizobial* strain.

D-4.1.6.4 Slide agglutination test

Mix 0.05 ml of culture broth with one drop of 1/100 diluted antiserum on a microscope slide and allow to react for 30 min. Observe agglutination under low power.

D-4.2 Quantitative Test

D-4.2.1 Viable or Plate Counts

Serially dilute one millilitre of the broth to obtain dilutions of the order of 10^6 to 10^9 . Plate 0.2 ml aliquots of the dilutions on YEMA plates and incubate at $28 \pm 2^\circ\text{C}$ for 2 to 6 days, depending on the species of *Rhizobium*. The counts of viable *Rhizobium* in the final broth from shake culture or fermentors shall be not less than 10^8 to 10^9 cells/ml. Otherwise, the broth should be rejected.

ANNEX E

(Clause 5.2.2)

DIRECTIONS FOR USE OF RHIZOBIUM INOCULANTS

E-1 The contents of the packet are sufficient for seeds to be sown in the area indicated on the package.

E-2 Use only for the leguminous crops mentioned, before the expiry date and do not expose to direct sunlight or heat.

E-3 Mix the inoculants with the seeds gently with

the minimum amount of water, taking care to avoid damage to seed coat. Dry the inoculated seeds under shade over clean paper or gunny bag and sow immediately.

E-4 This is not a chemical fertilizer and hence do not mix inoculated seeds or RI with chemical fertilizers.

ANNEX F

(Clause 6.1)

SAMPLING OF RHIZOBIUM INOCULANTS**F-1 GENERAL REQUIREMENTS**

F-1.0 In drawing, preparing and handling the samples, the following precautions and directions shall be observed.

F-1.1 Sampling shall be carried out by a trained and experienced person as it is essential that the sample should be representative of the lot to be examined.

F-1.2 Since the samples are also required for microbiological analysis, utmost care is necessary to avoid extraneous contamination while drawing and handling the samples and to preserve them in their original condition till they are ready for examination in the laboratory.

F-1.2.1 No preservative or bactericidal/fungicidal agent shall be added to samples required for microbiological analysis.

F-1.3 Samples in their original unopened packets should be drawn and sent to the laboratory. This will prevent possible contamination of the samples during handling and also help in revealing the true condition of the material.

F-1.4 Intact packets shall be drawn from a protected place not exposed to dampness, air, bright light, dust or soot, and transferred to clean containers.

F-1.5 The sampling appliances and sample containers shall be clean and sterile.

F-1.6 All precautions shall be taken to protect the sample the material being sampled, the sampling instruments and the sample container against adventitious contamination at the time of drawing the

sample, opening containers and transferring the samples.

F-2 SAMPLING EQUIPMENT

F-2.1 A suitable scoop made of stainless steel may be used for drawing samples for microbiological and modulation studies.

F-2.2 The sampling equipment shall be perfectly clean and sterile. It shall be properly sterilized by heating in a hot air-oven at 160°C for not less than 2 h or by autoclaving for not less than 20 min at 120°C and held in suitable containers to prevent re-contamination.

F-3 SCALE OF SAMPLING**F-3.1 Lot**

All units (containers in a single consignment of type of material belonging to the same batch of manufacture) shall constitute a lot. If a consignment consists of different batches of the manufacture the containers of the same batch shall be separated and shall constitute a separate lot.

F-3.2 Batch

An inoculant prepared from a batch fermentor or a group of flasks (containers) constitute a batch.

F-3.3 For ascertaining conformity of the material to the requirements of the specification, samples shall be tested from each lot separately.

F-3.4 The number of samples drawn from the lot shall depend upon the size of the lot and shall be according to Table I.

Table 1 Scale of Sampling
(Clause F-3.4)

No. of Packets in the Lot	For Requirements given in 4.1, 4.2, 4.4, 4.5 and 4.7	Sample Size for Nodulation given in 4.6	
(1)	(2)	(3)	(4)
100 to 1 000	5	0	2
1 001 to 5 000	8	0	3
5 001 and above	13	1	4

F-3.4.1 These packets shall be selected at random and in order to ensure the randomness of selection, procedures given in IS 4905 may be followed.

F-4 NUMBER OF TESTS AND CRITERIA FOR CONFORMITY

F-4.1 Each of the packets selected from the lot according to col 1 and 2 of Table 1 shall be tested for the requirements given in 4.1, 4.2, 4.4, 4.5 and 4.7 of the specification. For this purpose, test samples shall be prepared from each of the packets sampled from

the lot. A sample packet failing in one or more of these requirements shall be considered as defective.

F-4.1.1 The lot shall be considered to have satisfied these requirements if the number of defectives found in the sample is less than or equal to the corresponding acceptance number given in col 3 of Table 1.

NOTE — The purchaser may ask for the test certificate of the manufacturer to verify the requirement of viable *Rhizobium* cells/g of the carrier on dry mass basis, as given in 4.2 of the specification.

F-4.2 The lot having been found satisfactory according to F-4.1, shall be finally tested for effective nodulation as given in 4.6 of the specification. For this purpose, the number of packets given in col 3 of Table 1 shall be taken afresh from the lot and test sample prepared from each of the packets for effective nodulation.

F-4.2.1 The lot shall be declared as conforming to the requirements of the specification if none of the packets in the sample fails in effective nodulation.

F-4.3 The lot shall be declared as conforming to the requirements of the specification, if F-4.1 and F-4.2 are satisfied.

ANNEX G
(Foreword)
COMMITTEE COMPOSITION

Soil Quality and Improvement Sectional Committee, FAD 2'

<i>Organization</i>	<i>Representative(s)</i>
Indian Council of Agricultural Research, New Delhi	DR J. S. SAMRA (<i>Chairman</i>) ASSISTANT DIRECTOR GENERAL (<i>Alternate</i>)
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G.B Pant University of Agriculture and Technology, Pantnagar	PROF AND HEAD (SOIL SCIENCE)
Haryana Land Development Corporation, Chandigarh	CHIEF TECHNICAL OFFICER
Indian Agricultural Research Institute, New Delhi	HEAD (AGRI CHEM AND SOIL SCIENCE) HEAD, DIVISION OF MICROBIOLOGY (<i>Alternate</i>)
Indian Institute of Soil Science, Bhopal	DR C. L. ACHARYA DR A. SUBBARAO (<i>Alternate</i>)
In personal capacity (H-1543 Chittaranjan Park, New Delhi)	DR S. K. GHOSH
Ministry of Agriculture, Delhi	CHIEF SOIL SURVEY OFFICER CHIEF SOIL SURVEY OFFICER, AIS (<i>Alternate</i>)
M/s Pyrites, Phosphates and Chemicals Ltd, Rohtas	GENERAL MANAGER (MARKETING) MANAGER (AGRICULTURE SCIENCE) (<i>Alternate</i>)
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BIS Directorate General	SHRI P. K. SARKAR, Director and Head (FAD) [Representing Director General (<i>Ex-officio</i>)]

Member-Secretary
MRS MADHULIKA PRAKASH
Director (FAD), BIS

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This Indian Standard has been developed from Doc : No. FAD 27 (942).

Amendments Issued Since Publication

Amend No.	Date of Issue	Text Affected

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